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A practical guide to DNA extraction, PCR, and gene-based DNA sequencing in insects

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Abstract

Molecular methods have been highly informative for inferring evolutionary relationships, implementing diversification and biogeographic range evolution analyses, and inferring the evolution of traits of interest. Although these molecular laboratory methods are widely used across the tree of life, there are specific protocols that are useful within taxonomic groups. For these reasons I present protocols I have successfully used for DNA extraction, polymerase chain reaction (PCR), and cycle sequencing for gene-based and targeted genome sequencing of insects. This practical guide is not exhaustive and I encourage scientists to use, modify, and share these detailed steps as needed for their taxonomic group of interest or specific scientific question.

Keywords: DNA extraction, PCR, gene, sequencing, insects, protocol.

Introduction

Molecular methods are powerful tools for inferring the evolutionary relationships/systematics across the tree of life and implementing further diversification analyses. As many entomologists are interested in using molecular methods to infer evolutionary relationships of their groups of interest, here I present practical methods for genomic DNA extractions, the polymerase chain reaction (PCR) to amplify target genetic/genomic regions of interest, and cycle sequencing for Sanger-based DNA sequencing (Figure 1). This how-to guide is not meant to serve as an exhaustive explanation of all the possible methods or techniques, but only to outline the methods that I have been able to successfully implement (as in Saux et al. 2003; Saux et al. 2004; Moreau et al. 2006; Moreau 2008; Moreau 2009; Moreau 2011; Moreau & Bell 2011; Sarnat & Moreau 2011; Moreau & Bell 2013).

An additional concern of entomologists it how to preserve specimens in the field for future molecular analysis. For a review and quantitative analysis of commonly used preservatives for insects for molecular methods, please see Moreau et al. (2013) and the references within. In short, high percentage ethanol (95-100% EtOH) is ideal, although propylene glycol can be an effective preservative for passive traps or when there are safety concerns regarding the use and/or transport of ethanol.

Lastly, although these methods have been successful under the conditions, which I have used them there will always be exceptions and steps to improve these protocols for your specific group of interest or research question. I encourage you to modify (and share widely) the protocols outlined below. In addition, although many scientists are moving toward next-generation sequencing to sample either the reduced genome or whole genomes to address questions in systematics and evolutionary biology, for many scientists targeted genome and gene-based Sanger sequencing will likely remain a tractable, affordable, and useful method for many scientific questions and to infer the phylogenetic relationships of understudied taxonomic groups.

DNA EXTRACTIONS USING QIAGEN DNEASY KITS

(Online: 15-November-2014; Print: 9-January-2015)

These are the instructions I use for DNA extractions of individual or parts of ants, but are useful for almost any insect or tissue (invertebrates to vertebrates). They are rather comprehensive as they were written so that someone with any level of laboratory experience can follow the protocol.

These instructions are for extracting DNA from 24 or fewer ant specimens (number determined by size of microfuge centrifuge capacity; increase accordingly if necessary, but I suggest starting with much fewer samples to limit the chances for mistakes) using the Qiagen DNeasy DNA extraction kit and tungsten carbide beads and extraction machine, but this protocol can be easily modified to accommodate other kits or physical cuticle disruption.

Each individual collection or specimen must have a unique number so you can associate the voucher (which should be deposited in a curated university or museum collection) with each DNA extraction. Voucher specimens can take the form of an individual separate from the individual sacrificed in the DNA extraction (this works wel for social insects from the same nest series) or a specimen missing a specific body region (i.e. single leg or all the legs from one side of the body) if you only have a single specimen. First create a list of the specimens from which you will be extracting DNA (use an extraction worksheet or write in your lab notebook). You must be very diligent to never mix up a number or tube as this will lead to confusing the DNA of one specimen for another specimen. If you ever realize you have done this, it may be necessary to throw out the entire DNA extraction. Be sure to note this on your extraction worksheet or lab notebook.

Before you begin the DNA extractions it is very important to make sure everything you will be using is DNA free.

- Get six 96 tube racks, forceps, small beaker, Petri dish, and anything else you use for the protocol and put under the UV light bench for 15 minutes or longer (the UV will glow bluish when turned on) or soak in a 10% bleach solution and then allow to dry overnight. The bleach and/or UV light destroys DNA that could contaminate your new extractions.
- Once you are ready to begin, thoroughly clean your lab bench with 10% bleach and ethanol. Be sure it is entirely dry before putting your extraction materials on the bench.
- When above materials are clean and DNA-free, bring them to the lab bench with new, gloved hands.
- Check that the hybridization “rotisserie” oven or oven with “centrifuge tube rocker” is turn on and set at 55-56˚C.

Next make sure you are set up to begin the DNA extractions.

- I find that if you stagger the tubes in the racks by putting eight tubes in the first row of the rack in positions 1, 3, 5, 7, 9, 11, 13, 15 and then skip a row and then add the next eight tubes in positions 2, 4, 6, etc. and then the last eight tubes in the last row in positions 1, 3, 5, etc. this will allow room for opening all the tubes without the lids getting too much in the way.
- As you open the 1.5mL tubes (now and in all future steps) be careful not to put your thumb or fingers on the inside of the lid (you can introduce contamination this way).
- Put the vials currently holding the specimens you intend to extract in one of the newly sterilized 96 tube racks in the order in which you have entered them on your extraction sheet.
- In the next sterilized rack put one clean, lid-closed 1.5mL tube for every specimen you will be extracting in the 96 tube rack. Number these tubes on the top using a lab pen (Sharpie, VW pen, etc.) with the same numbering system as the worksheet (does not have to be the collection code; can be a number 1-24, etc.)
- Next put the same number of Qiagen extraction filter tubes in the next 96 tube rack (you will have to peel each tube

from its individually sealed holder). Number these with the same system you numbered the 1.5mL tubes above (i.e. 1-24, etc.)

- In the next two 96 tube racks set up the same number of empty Qiagen collection chambers/tubes as the number of extractions you are doing.
- In the last rack place closed 1.5mL tubes (again the same number as you are extracting). It is ideal to label these with the "Tough-Spots" stickers, as this will prevent the number from being accidentally rubbed away, writing the collection code from your extraction sheet (not #1-24, etc., but the actual unique specimen collection codes). These numbers should be the same collection codes as found on the vials/tubes you are taking the whole specimens from and correspond to the collection locality data for specimen you are extracting.

You are now ready to begin the DNA extractions.

- Be sure to have a box of sterile lab wipes ready (i.e. KimWipes, etc.).
- Make sure all your tubes are always in the same order as your extraction worksheet/lab notebook. This will help you to minimize errors.
- Open each of the 1.5mL tubes you labeled #1-24 with the lids opened away from you again being careful not to let your thumb or fingers touch the inside of the lid (these are the vials you will be placing the specimen into and adding the Qiagen chemicals to leave from three hours to overnight).
- Fill your Petri dish with sterile PCR water.
- Fill your small beaker with 95% ethanol and place forceps in this beaker (you will use these to take the individual insect specimens from the tubes).
- Begin with the first vial of specimens. Take one single insect specimen out of the vial using the forceps and place in the Petri dish of water, insuring the size and depth of your dish is appropriate to fully submerge your specimen.
- Leave in the water for 10 seconds to remove any ethanol on the specimen from the collection/storage tube. No need to shake around.
- Remove the insect specimen from the PCR water using the forceps and place gently on a clean lab wipe.
- Leave on lab wipe for a few seconds to get most to all of the water off.
- Place the insect specimen in a labeled, open 1.5mL tube corresponding to the correct extraction number. Always check everything twice!
- Once you have placed an insect specimen in the open 1.5mL tube, leave the tube open to allow to the insect specimen to dry, but turn cap towards you to indicate that this vial now has an insect specimen inside (this will help you to not accidentally put two insects in the same vial).
- Clean forceps in the beaker of ethanol and wipe with a clean lab wipe.
- You are now ready to begin with the next specimen.
- Continue until all insect specimens are in an individual 1.5mL tube.

With one single insect specimen in each tube, you are now ready to move to the next step.

- If using the Qiagen tungsten carbide bead and Qiagen extraction machine use the following steps, otherwise physically homogenize the specimen with a sterile Teflon pestle in the first extraction kit solution (both methods work well):
- Again clean your forceps in the beaker of ethanol and wipe clean with a lab wipe.
- Get a vial of clean Qiagen tungsten carbide beads (or a clean pestle if you do not have Qiagen tungsten carbide beads).
- Place one bead in each tube with the insect specimen and close the tubes.

- Load the vials with the insect specimen and tungsten carbide bead into the Qiagen extraction machine (be sure to load both racks equally – they must be balanced) (or grind each specimen with a clean pestle).
- You will turn on machine for 20 seconds (no more than 20 seconds) at 30.0 1/s frequency.
- Take the tubes with the now shattered ant with the metal bead inside and place back in the rack in numerical order (again staggering the tubes in the rack).

You will now begin to follow the Qiagen DNA extraction instructions (be sure to always use barrier/filtered tips for pipetting).

- Open all the tubes (again with the lids open away from you).
- As you are opening each tube, check the lid to see if the metal bead cracked the lid (this happens rather frequently). If a tube has a cracked lid, carefully cut off the lid at the plastic hinge making sure not to lose the material inside after you have added the buffer and ethanol (outlined below) and vortexed, etc. Then cut the lid off a new tube and place on this tube. Be sure to label with the same exact number that was on the tube before you cut the lid off.
- Add 180uL of Buffer ATL to each tube.
- Add 20uL Proteinase K (ProK; >600 mAU/ml) to each tube (pipetting the mixture up and down), closing each tube as you finish this step.
- Once you have added the Buffer ATL and ProK to each tube, place each tube in the hybridization oven at 55-56˚C.
- Turn "rotisserie" or "rocker" on so the tubes are rotating or rocking while they are "cooking" at 55-56˚C. If you do not have an oven that can slowly rotate or rock your samples, you can physically shake the samples every half hour or so to insure mixing during this step.
- Leave tubes in the hybridization oven for three hours minimum to overnight (but not more than 30 hours as your samples may begin to evaporate).

After three hours to overnight, you are ready to finish the DNA extractions.

- Remove the tubes from the "rotisserie" and place back in the 96 tube rack.
- Get a small Erlenmeyer flask from the glassware cabinet (you will place the "dirty" metal extraction beads in here).
- Now follow the instructions in the Qiagen kit.
- Add the Buffer AL to each tube as per the instructions and vortex. Then add the 100% ethanol as instructed and vortex. After you pipette off this mixture (using a barrier/filtered tip) and add to the Qiagen filter containing extraction tubes, dump the "dirty" tungsten carbide bead in a small Erlenmeyer flask (you will clean these when you are finished the DNA extractions).

Notes about the Qiagen extraction kit:

- Always spin your tubes in the centrifuge with the hinges angled down. You must always put your hinges in the same orientation in the centrifuge to ensure maximum DNA recovery.
- On the last step, only add 200uL Buffer AE once. Do not do this step a second time as suggested in the instructions.
- Also once you have added the 200uL Buffer AE, wait 10 minutes before spinning in the centrifuge for the final step. This insures that maximum DNA is removed from the column.
- When spinning in centrifuge for final spin with collection barrier tubes placed within labeled final tubes, only put a tube in every other centrifuge hole and make sure the caps of the final tubes are not overlaid or they will snap off. If this occurs make sure you know which caps belong to which samples before taking any of the tubes out of the centrifuge.

Cleaning the Qiagen tungsten carbide beads.

- UV or bleach the forceps and a container for 15 or more minutes to hold

the metal extraction beads in the last steps (the plastic lid of a 96 well plate will work or the lid of an empty tip box).

- After placing all the "dirty" metal extraction beads in the small Erlenmeyer flask, add some distilled water and swish around. Get an empty beaker to pour into. As you will want to pour off the water and not lose any of the metal beads, pour this into the beaker so that any that fall out land in the beaker and not down the sink.
- Once you have poured off as much water as possible without pouring the metal beads out, get three lab wipes and fold in half and place over mouth of Erlenmeyer flask. Then dump over so the lab wipes to absorb the excess water (repeat if necessary, but beads do not have to be completely dry).
- Next add 0.4M HCL to the Erlenmeyer flask and "dirty" metal beads filling about ¼ full.
- Agitate in the solution for about 1-2 minutes to clean metal extraction beads (some may appear tarnished, but this is fine).
- You must pour this 0.4M HCL into a properly labeled waste jar under a fume hood. Again be careful to not lose the beads (you can leave a little of the HCL in the Erlenmeyer flask if you are worried you will lose the beads).
- Now add a little distilled water and again swirl around to wash the beads. Carefully pour off water making sure to not lose the metal beads in sink. Again use three folded lab wipes over the mouth of the Erlenmeyer flask to absorb last of the water.
- Now line the container (a sterilized empty tip container will work fine) with several lab wipes. Dump the beads in and rub them with another clean lab wipe.
- Now put container and metal extraction beads under the UV light for 15 minutes if available, otherwise soak them in 10% bleach solution for 5 minutes (or longer) insuring they are completely dry before proceeding to the next storage step. Every 5 minutes or so roll the beads around to expose all sides to the light.
- Once done, using forceps place beads in a new, clean 1.5mL tube. Only put 24 metal beads in each tube so they are ready for your next round of DNA extractions.

Special notes:

- If you ever are worried that you have touched something that can possibly contaminate your samples, always throw away your gloves and get new ones.
- Always use barrier/filter tips for DNA extractions (remember always use barrier/filter tips for any lab work up to and including PCR).
- It is best to do all DNA extractions in clear (non-colored) tubes. This allows for the visualization of contaminants in your tube such as pigments from the insect specimens or other items.
- If you are worried you may have ruined or contaminated an individual tube or sample, make a note of it on the worksheet. This will help narrow down potential problems later.
- Many of the Qiagen buffers have very similar initials/names, so be extra careful you are using the correct buffer in the correct order.
- Remember there is no such thing as being too careful in the lab.

Storage of your DNA extractions:

If you are using your DNA extractions often, it is best to store them in a standard refrigerator (~4˚C). For long-term storage it is best to place your DNA extractions in a non-frost free freezer (as these maintain the lack of frost by slightly warming and cooling the freezer) or deep freezer. Remember that repeated freezing and thawing your DNA samples can result in shearing of the DNA strands.

DNA EXTRACTION USING PHENOL/CHLOROFORM

The grinding solution is made from the following stock solutions:

	1 Sample	10 Samples
Water (PCR Grade)	305µL	3.05mL
0.5 M EDTA	100µL	1.0mL
0.1 M Tris (pH 8.0)	50µL	500µL
20% SDS	25µL	250µL
Proteinase K (20mg/ml)	20µL	200µL
Total	500µL	5.0mL

- Homogenize sample with Teflon pestle in grinding solution.
- Incubate the homogenate at 55°C for 3 hours (or overnight).
- Add 550µL of equilibrated phenol to the sample.
- Vortex for 1 minute. Microfuge for 5-10 minutes.
- Take off the supernatant (which contains the DNA) and place in new tube.
- Discard old tube or save to ensure DNA was not lost.
- Repeat steps 1-3 about 2-3 times, or until the supernatant is not cloudy and discolored.
- Add 550µL of chloroform to each sample.
- Vortex for 1 minute. Microfuge for 5-10 minutes.
- Take off the supernatant (which contains the DNA) and place in a new labeled tube.
- Discard old tube. Repeat steps 5-7 again (optional).
- Add 750µL of cold 100% EtOH to the supernatant and let stand for 2 hours at -20°C (can be overnight).
- Microfuge for 10 minutes to pellet the DNA and gently discard the supernatant by slowly pouring it off, leaving only the pellet.
- Gently add 50µL of cold 70% EtOH to the pelleted DNA.
- Microfuge for 10 minutes and gently discard the supernatant by slowly pouring it off, leaving only the pelleted DNA. Repeat steps 10 and 11 again.
- Dry pelleted DNA in speedvac or invert and allow to dry completely.
- Resuspend the dry pelleted DNA in 200µL 0.1 mM Tris pH 8.0 or 200µL PCR water.

PCR PRIMER SELECTION

Selection of genes/genomic regions for amplification and DNA sequencing and the PCR and cycle sequencing primers that are most appropriate will depend not only on your taxonomic group of interest, but also on the evolutionary age or depth of the group. I encourage you to read through studies related to your taxonomic group of interest and potentially investigate the genomic structure of any available genomes available to determine the appropriate level of molecular variation for your taxonomic group and the amount of molecular variation necessary to answer your scientific questions.

POLYMERASE CHAIN REACTION (PCR)

Most of the protocols to set up your PCR reaction are highly similar to those outlined below in the cycle sequence reaction section. You will need to vary the size of your reactions depending on the number of the samples you have extracted for amplification. I will not outline in detail how to set up a PCR reaction here since most students have the opportunity to do this during their studies. I will present the specific parameters I have used for insects, but if you need additional detail you can coopt the details below in the cycle sequence reaction section. Keep in mind if you add other chemicals to improve the efficiency of your chemical reaction you will need to reduce the amount of water in the reaction to keep the reaction at the same volume. Also note that to accommodate pipette error, it is advisable to set up your master mix for one or two more reactions that you are actually doing.

PCR master mix protocol (25 μL per sample master mix solution):

Component	Amount
Water (pure, DNA free water)	to 25 μL
10x buffer	2.5 μL
$MgCL_2$ (25 mM)	1.5 μL
dNTPs (8 mM)	2.5 μL
Forward Primer (10μM)	1.2 μL
Reverse Primer (10μM)	1.2 μL
DNA Template	1 μL
Taq polymerase(5U/μL)	0.1-0.2 μL (or less)

PCR thermal cycler parameters:

Step	Parameters
Step 1: Initial Denature	94˚C for 1 minute
Step 2: Denature	94˚C for 30 seconds
Step 3: Anneal	varies, but usual 48-58˚C for 1 minute
Step 4: Extension	72˚C for 2 minutes
Repeat steps 2-4:	30 times
Step 5: Final Extension	72˚C for 3 minutes

Storage of your PCR products:

Although not particularly temperature sensitive once your PCR is complete, it is best to store your PCR product in a standard refrigerator (~4˚C) to prevent evaporation.

PCR TROUBLESHOOTING

If your PCR sample does not amplify (or amplifies very weakly) try these steps in order:

- If your sample does not work at all the first time, try again with the exact same parameters and protocol.
- If your sample still does not work at all or amplified very weakly, next lower your annealing temperature by 2-5˚C (i.e. If you originally amplified at 54˚C try re-amplifying at 52˚C), but keep all else the same.
- If lowering by 2˚C does not work, try lowering the annealing temperature again by another 2-3˚C.
- If lowering the temperature does not work, try adding more $MgCl_2$. Add 1μL more $MgCl_2$ per reaction.
- In some rare cases with nuclear genes, raising (not lowering) the annealing temperature actually helps. Try raising the temperature by 2-5˚C from the original annealing temperature.
- You may also consider including an additive in your PCR (or changing the additive if you are currently already using one). Examples include BSA, DMSO, etc.

If a sample is not amplifying for multiple genes using the steps above:

- First try adding additional DNA template to your PCR reaction (i.e. if you originally were using 1μL per reaction, try adding 2-4μL per reaction).
- If you have reason to believe there is too much DNA in that particular extraction (i.e. this was the largest of your ants, beetles, muscle tissue, etc.), dilute a subsample of your DNA extraction by half with PCR water (i.e. 20μL original DNA extraction and 20μL PCR water) and use this as the template for the PCR.
- If the steps above do not work, re-extract that sample if you have additional material available.

If your PCR sample amplifies but sequences poorly in both directions:

- Re-PCR with a higher annealing temperature. If the samples still amplifies well (bright band on gel) try sequencing this product.
- If after amplifying with a higher annealing temperature this does not improve your sequence quality but your samples still amplify, then try raising the temperature again.
- If you still have low quality sequences, try reamplifying with less $MgCl_2$.

Special notes:

- You should always use barrier/filter tips for all PCRs if possible to reduce the amount of possible cross contamination.
- To get strong clean DNA sequences you must start with good PCR product.
- Remember lowering the annealing temperature and adding $MgCl_2$ both decrease the specificity of your reactions, so this can lead to amplifying

non-target regions of the genome or contaminates.
- Never amplify your PCRs below an annealing temperature of 45°C.
- Again remember there is no such thing as being too careful in the lab.

GEL ELECTROPHORESIS AND PCR CLEANING

Before proceeding to the cycle sequencing step, it is highly advisable to use gel electrophoresis to visualize your PCR product. This insures that you have 1) successfully amplified a product; 2) amplified the target PCR product size; and 3) permits confirmation of a single amplified PCR product (as long as the non-target product size is significantly different in length).

In addition, you must clean your PCR product to remove residual primers and unincorporated nucleotides. There are many commercial products available to complete this step.

CYCLE SEQUENCING, CLEANING, AND LOADING SAMPLES IN A SINGLE 96-WELL PLATE

To save time, money and lab supplies, you can cycle sequence your samples in the plate you are will actually load into the sequencing machine.

Cycle sequencing master mix protocol for each direction/single primer (10 µL per sample master mix solution):

5x buffer	1.65 µL
BigDye Terminator (BDT)	0.75 µL (or less)
Primer (10µM)	0.3 µL
DNA Template	1-4 µL
Water	to 10 µL

Cycle sequencing thermal cycler parameters:

Step 1:Initial Denature	96°C for 1 minute
Step 2: Denature	96°C for 10 seconds
Step 3: Anneal	50°C for 5 seconds
Step 4: Extension	60°C for 4 minute
Repeat steps 2-4:	25 times

To set up your cycle sequence reaction:
- Start with a clean 96-well plate (this can be a plate that has been used previously, but should be completely clean and dry before you begin.)
- You will need a silicon 96-well mat to cover your samples when you are finished, which should also be clean and dry (these mats can be used over and over).
- Make sure you have all your cleaned PCR samples ready (don't forget to quickly spin these down if they have been in the refrigerator, as there will likely be condensation on the lids).
- Put your 96-well plate on ice (only to prevent evaporation of your DNA template – the cleaned PCR product - once you load them into the wells of your 96-well plate).
- Make up your cycle sequencing master mix minus the Big Dye Terminator (BDT), which you should leave in the freezer until you are ready to use it since BDT is light sensitive. You will add the BDT at the very end to the master mix, but after you have your template in the wells.
- Put your master mix minus the BDT on ice.
- Add your DNA template (the cleaned PCR product) to each well of your 96-well plate. Be sure to load the plate from 1A – 1H then to 2A – 2H, 3A – 3H, etc. ending on well 12H.
- Tip – I usually add the template to the front inside of the tube/well, touching the pipette tip to the inside front side of the tube to insure the droplet of template does not stick to the outside of the pipette tip.
- Once you have your DNA template in each of the wells, get your BDT out of the freezer and add it to your master mix. Be sure to mix well by pipetting up and down several times.
- If you have added your template to the front inside of the wells, then you can

add your master mix to the back inside of each well, allowing the pipette tip to touch the inside of the well without chance of contamination.

- After you have added the master mix to each well, cover with the clean, dry silicon mat and quickly spin down your plate in the plate centrifuge to insure that your DNA and master mix are both in the bottom of the wells (be sure you have another mat covered plate to use as a balance).
- After placing in the thermal cycler, be sure to put out a box with aluminum foil to cover your plate, as BDT is light sensitive.

Storage of your cycle sequence reactions:

Although not particularly temperature sensitive cycle sequencing is light sensitive. For this reason it is best to wrap your samples in aluminum foil to protect them from light. Once your cycle sequencing is complete, you can leave them in the refrigerator for up to a week before cleaning and Sanger sequencing, as long as it remains in the dark.

Cleaning your cycle sequence reaction (EtOH/EDTA precipitation):

- Before you begin, quickly spin down your plate (making sure to balance the centrifuge) as there is likely condensation on the lid/mat. Note orientation of mat on 96-well plate so you can put it back on in the same orientation.
- Make a master mix of the EtOH/EDTA solution in a plastic trough so you can use a multi-channel pipette to dispense this solution into your 96-well plate. For a 10µL reaction, you will need to add 30µL 100% EtOH and 2.5µL 125mM EDTA, so for a 96-well plate make up the following:

  3300µL 100% EtOH

  275µL 125mM EDTA
- Mix solution by pipetting the solution up and down a few times.
- Add 32.5µL of the EtOH/EDTA solution to each well of your 96-well plate using a multi-channel pipette (you will only need to use one set of pipette tips if you do not touch the tips to the 96-well plate).
- Seal 96-well plate with silicon mat (again paying special attention to place the mat back on the tray in the same orientation as you took it off) and quickly vortex to mix.
- Leave at room temperature for 10-15 minutes in a dark location (i.e. in a drawer) or covered in aluminum foil.
- Spin in refrigerated (if available) centrifuge at 2500g for 30 minutes at 4°C. Be sure to balance centrifuge.
- Important: Proceed to next step immediately (you must be ready and present as soon as the centrifuge stops). If not possible, you must spin the sample again for 10 minutes to re-pellet the product.
- Remove silicon mat (again noting orientation of mat on 96-well plate) and invert tray onto folded paper towel and place in centrifuge rack.
- Place tray inverted into centrifuge and spin 50g for 2-3 minutes.
- Add 30µL 70% EtOH to each pellet (just eyeball pouring 70% EtOH into trough to use multi-channel pipette to distribute). No need to mix or vortex.
- Seal 96-well plate with silicon mat (again in same orientation as original cycle sequence reaction).
- Centrifuge plate 2000-3000g for 15 minutes at 4°C.
- Important: Proceed to next step immediately (you must be ready and present as soon as the centrifuge stops). If not possible, you must spin the sample again for 10 minutes to re-pellet your product.
- Again, remove silicon mat and invert tray onto folded paper towel and place in centrifuge rack.

- Place tray inverted into centrifuge and spin 50g for 2-3 minutes.
- Place 96-well plate in 65°C oven for 10 minutes to allow to thoroughly dry.
- While waiting, clean gray septa mat cover and make sure it is completely dry.
- If you cannot load samples directly onto sequencing machine at this time, you can cover the 96-well tray with a clean silicon mat, wrap in foil and freeze until you are ready to sequence.

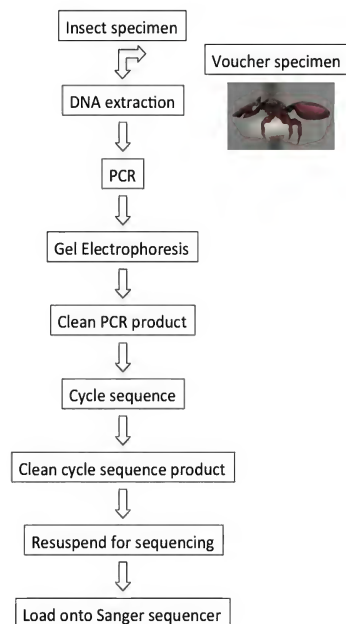
Resuspending samples in HiDi for sequencing

- Add 10µL to each well of the 96-well plate (if you have "blank" lanes without any DNA product, you must still load with HiDi or ddH_2O – there must be HiDi or ddH_2O in every well so as to not damage the capillary arrays of the sequencing machine).
- If your plate will be the first reaction on the sequencing machine, you must wait 10 minutes before placing your plate on the machine to allow for the pellet to resuspend in the water (if there are other plates ahead of your plate, you can place it directly into the queue).

Additional cycle sequencing notes:

- You do not need to use barrier/filter tips for cycle sequencing, except when you take aliquots from your stock reagents (like your primers) that are still used in your PCRs.
- For most cycle sequence reactions, there is no need to use more than 0.75µL of BDT per 10µL reaction (in some cases you may even be able reduce the amount of BDT to 0.50µL or 0.25µL per reaction). This will save you a substantial amount of money in the long run.
- If you only need to sequence a half plate, you must load your 96-well plate every other column (i.e. 1A – 1H, 3A – 3H, 5A – 5H, etc. ending on 11H), to insure the capillaries on the Sanger sequencer are immerged in the wells that contain your product.
- You can reuse your 96 well plates. Be sure to clean them thoroughly after they come off the sequencing machine, this includes washing with water and sterilizing when you have this option available. I have reused the same 96-well plates for more than year without any adverse effects.

Figure 1.Overview of the major steps of DNA extraction, PCR, and gene-based DNA sequencing in insects. Photograph of voucher specimen of Cephalotes varians by Gracen Brilmyer.



Conclusions

Molecular and genomic data provide a powerful and independent data source to address questions regarding the evolution of morphological characters, biogeography, diversification, and evolutionary relationships. My hopes with this detailed guide is to arm

scientists the world over to feel comfortable with the laboratory research protocols required to generate these data. In closing I hope this practical guide provides the foundation for future entomologists to leverage the power of DNA, genetics, and genomics to address questions in systematics and evolution.

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